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Effect of Hydrazines on Substrate Utilization by a Strain of *Enterobacter cloacae**

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Hydrazine and several of its methylated derivatives have been the subject of extensive investigations for nearly eight decades (UNDERHILL & KLEINER 1908, WEIR et al. 1964, CLARK et al. 1968, BACK et al. 1978, SHANK et al. 1979, HUNT et al. 1981, HENDERSON et al. 1981). The application of short-term in vitro toxicity test systems to the study of xenobiotic molecules (BERKY & SHERROD 1977) suggested consideration of the response of bacterial systems to hydrazines. LONDON (1979) showed the toxicity of hydrazine (Hz), monomethylhydrazine (MMH), and 1,1-dimethylhydrazine (UDMH) to a strain of soil bacteria to be comparable to that observed in other biological systems. A subsequent study by MANTEL & LONDON (1980) suggested different mechanisms of toxicity for Hz and MMH as indicated by growth rates of cultures grown previously in the presence of one of the compounds. During a series of preliminary experiments on the death rate kinetics of Hz exposed cultures, we noted growth in substrate-free media that suggested metabolites derived from lysed cells were being metabolized. This observation in conjunction with reports on the effects of Hz intoxication on carbohydrate metabolism (UNDERHILL & HOGAN 1915, IZUME & LEWIS 1926-1927, SMITH 1965, TAYLOR 1966, GEORGE & BACK 1977) prompted an investigation of the effects of Hz, MMH, and UDMH on the utilization of arbitrarily selected saccharides and compounds involved in intermediary metabolism as a means of elucidating mechanism of action.

MATERIALS AND METHODS

Culture methods and media. The organism used was Enterobacter cloacae strain D-31 isolated from soil and maintained at 4°C on slants of Tryptic Soy Broth (Difco) solidified with 1.5% Bacto-Agar (Difco). The mineral salts medium (SMS) was prepared as described previously (MANTEL & LONDON 1980) in Nephelo Culture Flasks (Bellco) and sterilized by autoclaving. Inocula, 0.1 ml/100 ml of medium, were obtained from 16 hr cultures, grown in SMS plus 2.0 g/L glucose, incubated at 20 ± 1°C on a reciprocating shaker (100 oscillations/min), washed twice in SMS, and adjusted with SMS to a cell concentration equivalent to 40% transmittance (T) at 570 nm (Coleman Junior Spectrophotometer). Substrates were sterilized by membrane filtration (0.2 µm, Millipore Corp.) and added aseptically to the Nephelo flasks several days prior to initiation of the experiment. The hydrazines were added to the flasks immediately before inoculation. The inoculum for the study of substrate utilization by Hz adapted cells was obtained by

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growing D-31 in SMS plus glucose containing 10 ppm (µl/liter) Hz. After 46.5 hr incubation at 20 ± 1°C on the reciprocating shaker, the adapted cells were aseptically washed and resuspended in SMS (without glucose), and adjusted to a turbidity equivalent to 40% T at 570 nm. Some experiments were conducted in 150 x 18 mm spectrophotometer tubes containing 15 ml of supplemented SMS, inoculated with 25 µl of washed cell suspension, and incubated at 20 ± 1°C on the shaker.

Substrates. The saccharide substrates were D-configuration and were added to the SMS medium to a concentration of 2.0 g/L. Glucose was obtained from Fisher Scientific; mannitol, maltose, and salicin from Difco, and all others from NBC. Substrates involved in intermediary metabolism were obtained from Sigma with the exception of sodium acetate and glycerol which were purchased from Fisher. The concentrations used, based on that selected for glucose (11 mM) and the number of carbon atoms in the molecule, were: glucose-6-PO_4, citrate, and isocitrate, 10 mM; succinate, malate, and α-ketoglutarate, 13 mM; glycerol, pyruvate, phosphoenolpyruvate, 3-phosphoglycerate, and lactate, 20 mM; and acetate, 30 mM.

Hydrazines. Hydrazine and MMH were purchased from Eastman Chemicals and dispensed in small glass bottles under nitrogen. UDMH was a redistilled sample from Air Force supplies and distributed in glass under nitrogen. All samples were sterile and were added directly to the flasks and tubes with sterile (Hamilton) microliter syringes. Based upon responses observed in previous studies (LONDON 1979) the concentrations used were: Hz, 10 ppm (v/v); MMH, 20 ppm; and UDMH, 50 ppm.

Growth assay. Responses of D-31 were determined turbidimetrically with a Coleman Junior Model 6D Spectrophotometer at a wave length of 570 nm.

RESULTS

The characteristic responses of D-31 to the selected concentrations of Hz, MMH, and UDMH when grown on glucose are presented in Figure 1. The observable consequences of exposure were an increase in the duration of the lag phase not attributable to death of inoculum cells (LONDON, unpublished data) and a decrease in cell density at stationary phase with MMH. Growth rates during the log phase were essentially identical. The control and UDMH cultures initiated log growth in approximately 8 hr, while MMH exposure required 24 hr incubation and Hz exposure required 28 hr.

Growth curves constructed from the turbidimetric data were used to determine a quantitative measurement of growth rate. Since the slopes of all curves were essentially the same in log phase, the measurement selected was the time to reach a level of growth equivalent to the approximate inflection point of the control curves (75% T for the tube cultures and 60% T for flask cultures). Data obtained from studies on the effects of hydrazines on saccharide utilization and on substrate utilization by

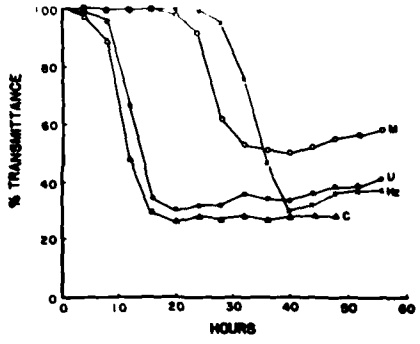

Figure 1. Effect of hydrazines on the growth of D-31 with glucose. Symbols: C, control; Hz, hydrazine; M, monomethylhydrazine; and U, 1,1-dimethylhydrazine

Hz-adapted cultures are presented in this manner (TABLES 1 and 2). The responses of D-31 on other carbon/energy sources are presented in Figure 2 as growth curves since differences in effects are more evident for these data when considered graphically.

Table 1. Effect of Hydrazines on Growth of D-31 with Various Saccharide Substrates

		Time in Hours to 75% T			
Substrate	Type*	C	H	MMH	UDMH
Galactose	M-6	14	39	27	18
Levulose	M-6	14	85	40	22
Mannose	M-6	14	60	36	18
Ribose	M-5	17	43	31	20
Xylose	M-5	18	91	39	34
Lyxose	M-5	<	NG	NG	<
Sorbitol	A-6	14	76	43	18
Inositol	A-6	44	NG	NG	74
Dulcitol	A-6	NG	NG	NG	NG
Maltose	D	13	87	42	21
Trehalose	D	12.5	85	36	14
Lactose	D	NG	NG	NG	NG
Turanose	D	NG	NG	NG	NG
Raffinose	T	16	NG	48	38
Inulin	P	NG	NG	NG	NG
Salicin	M	<	<	<	<

*M-6 = monosaccharide, 6 carbon; M-5 = monosaccharide, 5 carbon, A-6 = 6 carbon sugar alcohol; D = disaccharide, T = trisaccharide; P = polysaccharide; M = miscellaneous

†< = growth less than 75% T; NG = no growth.

The culture did not metabolize four of the saccharides studied - dulcitol, lactose, turanose, and inulin - and did not grow well on lyxose and salicin (TABLE 1). With the exception of the extended lag period with inositol (44 hr to 75% T), control cultures utilized the remaining saccharides with similar lag periods. Extension of the lag phase due to Hz exposure was more varied, ranging from an increase in lag phase by a factor of 2.5 for ribose to complete prevention of growth with raffinose, inositol, and lyxose. The degree of MMH inhibition was not as variable and less extensive than for Hz. The inhibitory effects of UDMH, with the exception of xylose and inositol, were minimal.

The effects of Hz, MMH, and UDMH on the growth of D-31 with various metabolites are shown in Figure 2. Several of the compounds selected for study (lactate, phosphoenolpyruvate, isocitrate, and 3-phosphoglycerate) did not support growth. The responses with α-ketoglutarate were not presented, since growth on this substrate was not as extensive as with glucose and in the presence of the hydrazine compounds did not differ significantly from control patterns.

Growth curves for the control cells indicate all substrates with the exception of acetate supported good growth of D-31. Lag periods for control cultures grown on most of the compounds were equivalent to that observed for glucose. Growth on succinate was marked by a somewhat extended lag phase (22 hr) while glucose-6-PO_4 was utilized more rapidly (12 hr lag) than glucose, as might be anticipated. Growth rates were quite similar and stationary growth phase was attained at about the same time regardless of treatment. Since these substrates were used at equivalent concentrations, observed differences in culture densities in stationary phase were probably attributable to differences in energy efficiencies.

Substrate utilization in the presence of UDMH (Figure 2) was similar to the control with the major exception of growth on citrate which resulted in a decreased final cell concentration and a marked tendency for cell lysis after 40 hr incubation. Except for succinate, final cell yields were similar for control and UDMH exposed cultures. Hz exposure resulted in an inhibition of the utilization of acetate, citrate, and glycerol. Of those substrates that D-31 could metabolize in the presence of Hz, glucose-6-PO_4 was most affected as indicated by the extended lag period of 42 hr. This is of interest since this substrate was utilized most readily and provided a high cell yield in the absence of Hz. Utilization of malate, succinate, and pyruvate was essentially unaffected by Hz except with some diminution in final cell yield. The curves obtained with MMH were very similar to the patterns observed with Hz with the significant exception that glucose-6-PO_4 metabolism was prevented while glycerol was actively utilized after an extended lag time. Growth rates during log phase for all of the substrates in the presence of the hydrazines were essentially the same.

TABLE 2 presents the responses of Hz-adapted D-31 with various substrates in the presence of Hz compared with the responses of

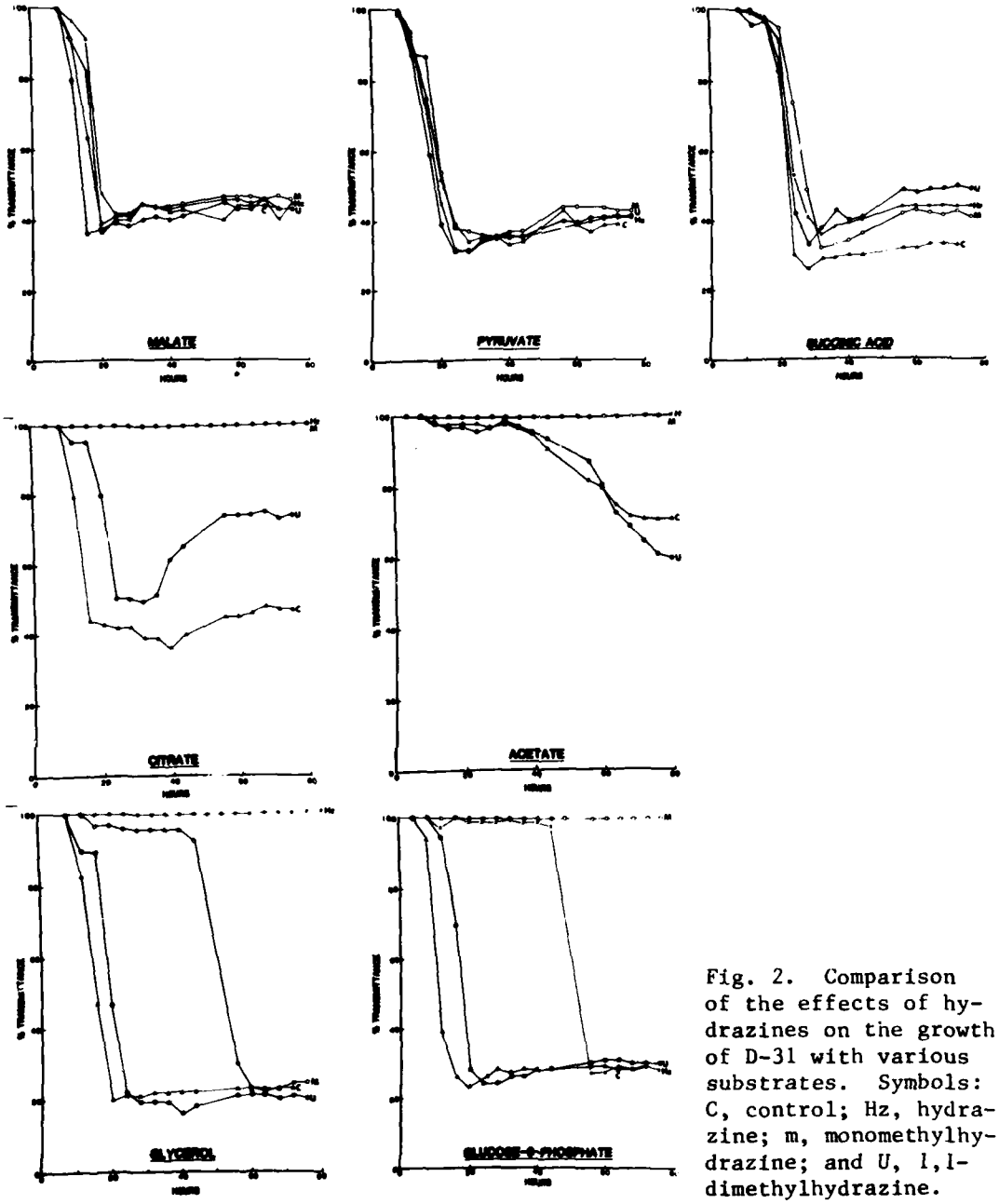

Fig. 2. Comparison of the effects of hydrazines on the growth of D-31 with various substrates. Symbols: C, control; Hz, hydrazine; m, monomethylhydrazine; and U, 1,1-dimethylhydrazine.

Hz-exposed, non-adapted cells, and control cultures. With glucose adapted cells in 10 ppm Hz grew almost as well as control cells. The extension of the lab phase, observed with non-adapted Hz-exposed cells (54 hr to 60% T), was reduced to 15 hr. Similar patterns were observed for galactose, levulose, and sorbitol, suggesting a similar adaptive mechanism for these four substrates. Maltose, trehalose, and mannitol growth responses presented similar patterns where adaptation occurred but to a lesser extent than with

Table 2. Growth of Hydrazine-Adapted D-31 on Various Substrates in the Presence of Hydrazine

	Time in Hours to 60% T		
Substrate	C	H	Adapted
Glucose	10	54	15
Galactose	10	36	16
Levulose	15	735	24
Ribose	18.5	37	25
Xylose	21	49.5	49
Sorbitol	10	62.5	22.5
Mannitol	13.5	47	31.5
Maltose	12	65	30
Trehalose	12	51.5	34
Raffinose	28	NG	72
Glycerol	18	NG	NG
Glucose-6-P	13.5	47	40

C = control; H = non-adapted, Hz exposed;
A = adapted, Hz exposed.

glucose. Growth with ribose indicated an adaptive response of the same relative extent as seen with maltose, trehalose, and mannitol, but not as much in absolute terms since the inhibition (increase in lag time) was not as great. The response for raffinose showed adaptation resulted in utilization of this substrate with significant delay (72 hr to 60% compared to 28 hr for the control) in contrast to the total prevention of growth by Hz-exposed, non-adapted cells. Adaptation of D-31 to Hz did not alter the growth response with xylose. This is also noteworthy since Hz inhibition of xylose metabolism is less than that with glucose as substrate as demonstrated by the difference in lag time between control and Hz-exposed cells of about 40 hr for glucose as compared to 24 hr for xylose. When Hz-adapted cells were grown with glucose-6-PO_4 as the carbon and energy source, some reduction in the inhibitory effect of Hz occurred. Adaptation, however, did not alter the response of D-31 with glycerol in the presence of Hz since the adapted cells were also not capable of growth under these conditions.

DISCUSSION

The ability of E. cloacae to utilize different substrates as sole carbon and energy source was variously influenced by the presence of a particular hydrazine compound. These effects ranged from reduction in final cell concentration to total inhibition of growth. The effects on substrate utilization were related to the specific hydrazine compound (at the indicated concentrations) with Hz having the greatest influence and UDMH the least, as has been observed previously (LONDON 1979). Attempts to discern an underlying mechanism of inhibition based upon similarity of structure or relationships in metabolic pathways did not prove fruitful. Of the saccharides metabolized by control cultures, Hz prevented the

utilization of the pentose lyxose, the trisaccharide raffinose, and the six carbon sugar alcohol inositol. MMH inhibited metabolism of lyxose and inositol and lyxose was also not used in the presence of UDMH. The extension of the lag phase in the presence of Hz occurred with all the saccharide substrates by varying amounts. In general, UDMH exposure resulted in the smallest increase in the duration of lag phase while MMH exhibited an intermediate effect. Thus, the consistency of effect was a result of the specific toxic compound and not the substrate.

The apparent lack of correlation of substrate structure with effect of the hydrazines was also observed with the responses to intermediary metabolites. None of the hydrazines affected the utilization of pyruvate, malate, or succinate (except for final yield) while citrate and acetate were not metabolized at all by Hz or MMH exposed cells. The most significant difference in the effects of Hz and MMH concerned the complete inhibition of glucose-6-PO_4 metabolism by MMH and the prevention of glycerol utilization by Hz. Thus, the growth response of D-31 with these compounds is an attribute of a specific substrate-inhibitor interaction. Since glucose was ultimately metabolized by MMH exposed cells and glucose-6-PO_4 was not, the inhibition by MMH did not occur in the glycolytic pathway.

Adaptation to 10 ppm Hz with glucose as substrate resulted in reducing the inhibitory effect of Hz on the utilization of the saccharides studied, with the exception of glycerol and xylose. SPAIN et al. (1980) showed that adaptation could result in an increase in the microbial degradation rates of selected organic compounds. The pioneering work of STANIER (1947) described the application of simultaneous adaptation as a technique for the elucidation of metabolic pathways. This approach is based on the fact that adaptation of a culture to a given substrate is characterized by simultaneous adaptation to other compounds that comprise the dissimilatory pathway in the utilization of that substrate by virtue of a complement of adaptive enzymes. Since the saccharide substrates examined in this study represent a disparate group and, at least initially, do not share common metabolic pathways, the adaptation observed (reduced inhibitory effect) may be associated with some other shared activity. As described by DILLS et al. (1980), five mechanisms that are carrier mediated are known to account for carbohydrate transport in bacteria. Various carbohydrates may be transported by more than one system or subsystem, e.g., xylose, glucose, and galactose can each function as substrate in more than one subsystem of the shock-sensitive transport system in Escherichia coli. Conversely, one system may be responsible for the transport of several substrates, e.g., the methyl-β-galactose shock sensitive system is effective in the transport of glucose, galactose, xylose, arabinose, fucose, and other substrates. Thus, inhibition of a given molecule such as Enzyme I, II, or III of the group translocation system could result in the prevention of utilization (transport) of several different substrates. Therefore, the data could be interpreted as indicating the sites of inhibition by hydrazines to be associated with active transport mechanisms, some

of which may be common to some of the substrates studied and others concerned with specific compounds, e.g., the transport of glucose-6-PO_4. Increased lag time could have resulted from interferences with the rates of transport across cell membranes, the three hydrazines affecting these rates differently. Further studies with cell-free systems should provide data for clarification of the inhibitory mechanisms.

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